Maintenance and Transmission of Keystone Virus by *Aedes atlanticus* (Diptera: Culicidae) and the Gray Squirrel in the Pocomoke Cypress Swamp, Maryland

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ABSTRACT Ecological studies were done in the Pocomoke Cypress Swamp, Worchester Co., Md., to clarify the role of *Aedes atlanticus* and gray squirrels as enzoötic hosts of Keystone (KEY) virus. Among 43 seronegative squirrels, 65% were infected with KEY virus during July, August, and September 1975, coincident with the emergence and activity of KEY virus-infected *Ae. atlanticus*. Minimum infection rates for this mosquito increased from 2.7 to 11.2/1,000 from July to October, but we could not conclude that KEY virus-infected squirrels served as an amplifying host. Although *Ae. atlanticus* was infected readily following per os exposure to KEY virus, only 1 of 83 infected mosquitoes transmitted virus to a suckling mouse. In contrast, 33 of 46 (72%) parenterally infected *Ae. atlanticus* transmitted virus to mice. KEY virus also was transmitted vertically by *Ae. atlanticus* to F_1 progeny, and one of two vertically infected females transmitted virus to suckling mice by bite. Preliminary data suggested that the per os-infected mosquitoes failed to transmit KEY virus because of a midgut escape, salivary gland barrier(s), or both.

KEY WORDS Insecta, Keystone virus, *Aedes atlanticus*, gray squirrel

ECOLOGICAL INVESTIGATIONS in the Pocomoke Cypress Swamp (PCS), Worchester Co., Md., demonstrated that Keystone (KEY) virus was transmitted vertically by *Aedes atlanticus* (Dyar and Knab), (LeDuc et al. 1975b). These data indicate that transovarial transmission can serve as an overwintering mechanism for this virus (LeDuc 1978). However, an epidemiological model proposed for KEY virus revealed that additional information was needed on the role of vertebrates to more fully assess the significance of vertical transmission in the maintenance cycle of KEY virus (Fine & LeDuc 1978).

Evidence from mosquito host preference studies (LeDuc et al. 1972) and serological surveys indicates that the gray squirrel (*Sciurus carolinensis*) has a more significant role in the transmission of KEY virus than other mammals on the DelMarVa Peninsula (LeDuc et al. 1975a, Watts et al. 1982). In addition, experimental infection of this species with KEY virus produced a viremia of 2–4 d duration, with maximum titers of 3.1–5.0 $\log_{10}$ suckling mouse intracerebral lethal dose $(SMICLD)_{50}$/ml (Watts et al. 1979), thus suggesting that gray squirrels are a virus-amplifying host. Therefore, we did field and laboratory studies to define further the respective roles of the gray squirrel and *Ae. atlanticus* in the maintenance and transmission of this California serogroup virus at the PCS.

Materials and Methods

Squirrel Capture and Specimen Collection. Gray squirrels were captured in National Live-traps (Tomahawk Live Trap Company, Tomahawk, Wisc.) (15 by 48 by 15 cm) in an oak and pine forest along the western edge of the PCS during 1975 through 1978. Traps were placed at each of 23 sites, 30–120 m apart. Corn on cobs was provided as bait to squirrels and was available ad lib. in rectangular hardwire baskets at a height of 4–5 m, attached to trees. Traps were set during the late evening and then checked the following morning and evening for 2–3 consecutive days per week. Captured squirrels were anesthetized with methoxyfluorane, bled by cardiac puncture, and marked with numbered ear tags. The sex, age (Brown & Yeager 1945), and location of capture were recorded, and squirrels were released at the capture

In conducting the research described in this report, the investigators adhered to the "Guide for the Use and Care of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council. The facilities are fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

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sites. Blood specimens were centrifuged at 400 × *g* for 15 min at 4°C. Sera were stored at −20°C until assayed for antibody. Blood cell pellets were stored at −70°C until assayed for virus.

Collection of Mosquitoes. *Aedes atlanticus* were collected with CDC miniature light traps (Hausherr's Machine Works, Toms River, N.J.) supplemented with dry ice. Traps were operated in the field on alternate nights from approximately 1800 to 0800 hours (EST) the following day, beginning 10 May and extending through 24 October 1975. The traps were suspended from tree limbs at a height of 1.5 m at six sties in the upland forest and at two sites in a swamp habitat. Mosquitoes were chilled briefly at −20°C, identified by species, placed in pools of 25 or fewer according to date and site of capture, and stored at −70°C until assayed for virus. Blood-engorged *Ae. atlanticus* were not assayed for virus. Population abundance estimates for *Ae. atlanticus* during 1976, 1977, and 1978 were based on larval surveys and the number of adults attracted to humans.

Serology and Virus Assays. Gray squirrel sera were assayed for neutralizing (N) antibody to Jamestown Canyon (JC) and KEY viruses by plaque reduction neutralization (PRN) tests in baby hamster kidney (BHK-21) cells, clone 15 (Watts et al. 1982). Sera were heat-treated for 30 min at 56°C and screened for antibody at 1:10 dilutions. Sera that reduced the virus dose by ≥50% were considered positive for N antibody. Selected sera, which neutralized JC and KEY viruses (≥50%), were diluted further to determine the plaque reduction 50% (PRN_{50}) titers from graphic plots on probit papers (Russell et al. 1967). Neutralizing antibody was considered specific for a particular virus if titers were 4 times or greater than heterologous viral titers.

Gray squirrel blood specimens were assayed for virus by intracerebral inoculation of 1- to 3-d-old albino mice (Watts et al. 1979). Mosquito pools were triturated in 5-ml Ten Broeck tissue grinders (Fisher Scientific, Pittsburgh, Pa.) in the presence of Medium-199, 20% heated fetal bovine serum (FBS), and antibiotics. One ml medium was used for pools containing 1–10 mosquitoes and 2 ml for pools with 11–25 mosquitoes. Mosquito suspensions were centrifuged at 800 × *g* for 30 min at 4°C and supernatant fluids were assayed for virus by intracerebral inoculation of 1- to 3-d-old albino mice (LeDuc et al. 1975c). Viral isolates were identified in PRN tests with JC and KEY virus–mouse hyperimmune ascitic fluid and prepared as described by Brandt et al. (1967).

Laboratory Transmission Trials. Female mosquitoes were collected from the PCS and used directly in experiments, or they were allowed to feed upon hamsters or rabbits to produce eggs for production of F_1 females. The oviposition substrates consisted of moistened sphagnum moss and multiple layers of moistened cheesecloth. Eggs were hatched by submerging them in a 1:1,000 dilution of nutrient broth in distilled water. The larvae were fed Tetramin staple fish food (Tetrawerke Company, West Germany) and finely ground guinea pig food pellets. Adult mosquitoes were maintained on 5% sucrose in an insectary at 26 ± 2°C, 65–85% RH, with a 16:8 (L:D) photoperiod before and during the experiments. Reared mosquitoes were 7–12 d old when used in the experiments.

The KEY viral strain used to infect mosquitoes was isolated from *Ae. atlanticus* adults collected in 1972 from the PCS (LeDuc et al. 1975c). This virus, used during 1975, had been passed twice in baby hamster kidney cell culture (BHK-21, clone 13) and underwent five additional passages in Vero cells before use in 1985 experiments.

Infectious blood meals consisted of virus-defibrinated guinea pig or chicken blood mixtures and were made available to mosquitoes through a lambskin membrane attached to a temperature-controlled feeding apparatus (Rutledge et al. 1964). The temperature of the virus–blood suspensions was maintained at 35–37°C. Mosquitoes inoculated intrathoracically with KEY virus received 0.18 μl/mosquito.

After mosquitoes ingested virus, they were retained either as a group or as individuals in the presence of an oviposition substrate. The per os and inoculated mosquitoes were allowed to refeed on 1- to 3-d-old mice for determination of virus-transmission rates. The inoculated mosquitoes were allowed to deposit second-ovarian cycle eggs to determine if virus was transmitted vertically. A sample of these mosquitoes was used to determine the distribution of virus in selected organs, and the remainder were tested as intact specimens to determine overall infection rates. The first- and second-ovarian cycle egg batches from individual inoculated females were stored separately in a humidified dessicator. Egg batches were hatched separately as described above, and larvae were either stored individually at −70°C or reared to adults for virus assay. These adults, males and females, were assayed individually to determine transovarial and filial infection rates. Females also were allowed to feed on mice to determine if transovarially infected mosquitoes were capable of transmitting KEY virus.

KEY virus transmission by *Ae. atlanticus* was based on the recovery of virus from brain tissue of mice that became ill or died after being fed upon by a mosquito. In addition, blood was obtained from the retro-orbital sinus of mice that survived for 21 d for antibody assay.

The KEY viral titer of the infectious blood meals and estimates of this virus in individual mosquitoes and selected organs were determined according to methods of Watts et al. (1972). Viral titers for mosquitoes were computed as described by Reed & Muench (1938). Confirmation of the identity of virus recovered from infectious blood meals, mosquitoes, and from brain tissue of mice that died during the experiments was established with mouse

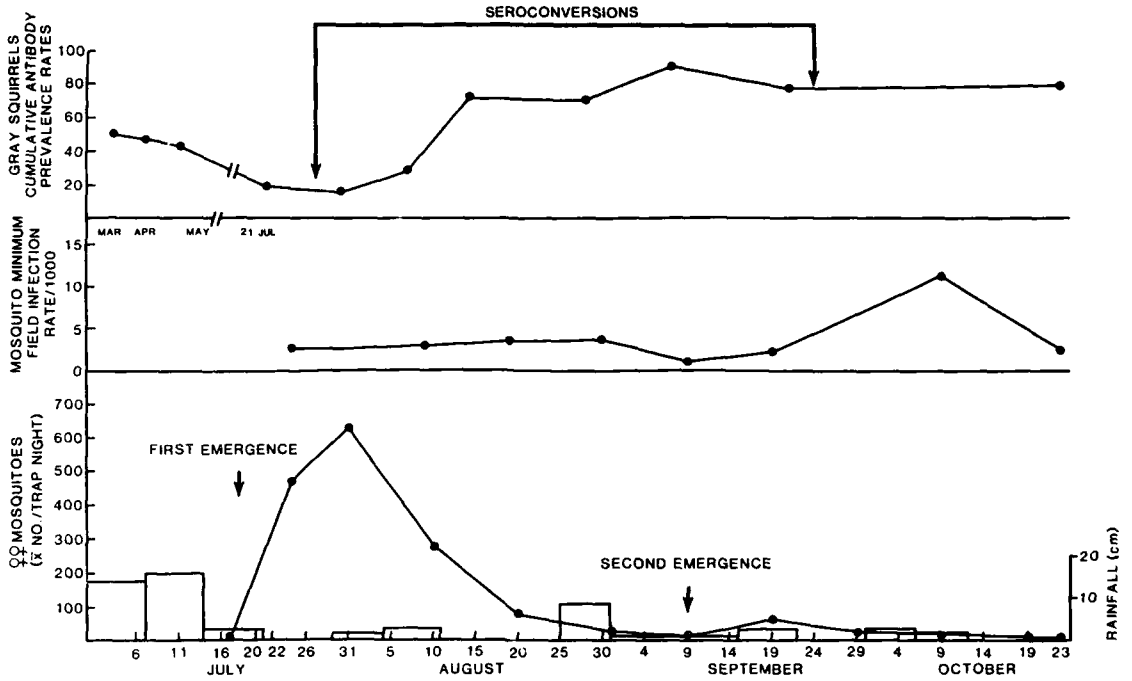

Fig. 1. *Ae. atlanticus* abundance relative to rainfall, bar graph (lower figure). The KEY virus minimum field infection rate for this mosquito (middle figure). The prevalence of KEY virus antibody in gray squirrels during the 1975 season (upper figure).

hyperimmune, KEY virus antisera in mouse neutralization tests and in PRN tests using BHK-21, clone 15 cells.

Results

KEY Virus N Antibody in Squirrels. KEY virus accumulative N antibody prevalence rates for gray squirrels sampled in 1975 at the PCS varied from 18 to 50% during March, April, and May; and then decreased to 19% ($n = 32$) and 16% ($n = 44$) during the third and fourth weeks of July, respectively (Fig. 1). Squirrels were not trapped during June. Antibody rates increased markedly during August, and by the first week of September, antibody was found in >90% ($n = 30$) of the squirrels. Attempts to capture squirrels after 4 October were unsuccessful.

The increase in KEY virus antibody prevalence in squirrels was correlated closely with the emergence of *Ae. atlanticus* on 17 July 1975 (Fig. 1). Ten days after the first adults were collected in the study area, three of eight previously seronegative squirrels acquired KEY virus N antibody. Subsequently, or after the peak *Ae. atlanticus* emergence, 65% ($n = 43$) of seronegative squirrels captured between the third week of July and the same week of September acquired KEY virus antibody. Among the 28 that seroconverted, 23 were adults and 5 were juveniles. Similar rates were found in males and females. The squirrels that did not seroconvert included nine adults and six juveniles.

Nine KEY virus antibody-positive squirrels captured during the spring of 1975 subsequently were recaptured during July and early August and were seronegative (Fig. 2). Of these, five apparently were reinfected, because all were seropositive when captured during late August.

The KEY virus antibody prevalence rate for gray squirrels decreased from 66% ($n = 88$) in 1975 to 10% ($n = 41$) in 1978 (Table 1). Of the 82 squirrels captured during 1976, 38% ($n = 82$) had been tested for KEY virus antibody during 1975. The antibody prevalence rate was 71% ($n = 31$) for the 1975 squirrels, and 31% ($n = 51$) for squirrels first captured in 1976. Virus transmission was low during 1976, as reflected by a single conversion among the 10 seronegative squirrels sampled, thus suggesting that most infections occurred prior to that year. Although 42% of the squirrels captured in 1977 and 10% in 1978 had antibody, serological evidence of newly acquired infection was not detected in 25 seronegative squirrels recaptured during this 2-yr period. Sera from two seropositive squirrels initially captured in 1977 reduced the KEY viral PRN dose by 99 and 59%. In 1978, sera from these same two squirrels reduced the viral dose by 28 and 0%, respectively. The marked decrease in KEY virus transmission subsequent to 1975, and the suggestive evidence of waning antibody titers, resulted in an overall reduction of antibody prevalence rates.

Specificity of Antibody. Sera from 10 of 88 squirrels sampled in 1975 completely reduced JC

Table 1. Age- and sex-specific KEY virus neutralizing antibody prevalence rates for gray squirrels, PCS

Year	♂♂			♀♀			Total
	Adults	Juveniles	Age unknown	Adults	Juveniles	Age unknown	
1975	73 (48)[a]	22 (9)	0	70 (23)	62 (8)	0	66 (88)
1976	56 (41)	9 (11)	60 (5)	60 (20)	0 (2)	33 (3)	49 (82)
1977	51 (35)	0	25 (4)	38 (21)	0	0 (4)	42 (64)
1978	12 (25)	0	0	7 (14)	0 (2)	0	10 (41)
Total	53 (149)	15 (20)	44 (9)	47 (78)	42 (12)	14 (7)	47 (275)

[a] Percentage positive (no. tested).

and KEY viral PRN dose when screened at the 1:10 dilution. However, the PRN_{50} titers were consistently higher against KEY virus as indicated by titers that ranged from 1:40 to 1:1,280 (geometric mean titer, 1:308) compared with titers that ranged from 1:10 to 1:130 (geometric mean, 1:37) against JC virus.

Virus was not isolated from 251 blood specimens obtained from gray squirrels captured between 16 July and 17 September 1975. Included among these were six seropositive squirrels that were seronegative when bled 4–6 d earlier.

Abundance of *Ae. atlanticus*. The initial emergence of *Ae. atlanticus* in 1975 was associated closely with rain (30 cm) received during the first two weeks of July. The first adults were collected on 17 July, 6 d after the first larvae were observed, and then peaked with an average of 611 mosquitoes per trap night during 27 July–5 August. After rain (9 cm) during the last week of August, a second hatch occurred, and the average number of *Ae. atlanticus* in light traps peaked at 64 per trap night during 15–24 September. Thereafter, the number collected declined to only nine mosquitoes per trap night, 15–23 October, the final night of trapping.

In 1976, *Ae. atlanticus* larvae were observed in the PCS after rains on 8, 15, and 28 August and 30 October; however, water in ground pools dried rapidly and precluded substantial adult emergence. During 1977, rainfall was insufficient to flood *Ae. atlanticus* oviposition sites. Again in 1978, *Ae. atlanticus* larvae were observed following rains during May through August, but few adults emerged. Annual monitoring of *Ae. atlanticus* at the PCS did not reveal a significant hatch of this mosquito until 1985.

KEY Virus in *Ae. atlanticus*. Keystone viral infection rates for 64,500 *Ae. atlanticus* collected during 1975 are presented in Table 2. The minimum infection rate (MIR) varied from 2.7/1,000, during 22–26 July, to 3.7 between 22 July and 4 September, and then declined abruptly to 1.0 during 5–14 September. Subsequently, the MIR increased significantly to 11.2/1,000 ($\chi^2 = 22.34$, $P < 0.025$) during 5–14 October, followed by a marked decrease to 2.7 during 15–23 October, or the final collection period of 1975. The final rate in October was similar to that observed for mosquitoes that emerged in July.

Laboratory Infection and Transmission of KEY Virus. The infection rates for *Ae. atlanticus*, which ingested different concentrations of KEY virus during 1975 and 1985 are presented in Table 3. As the

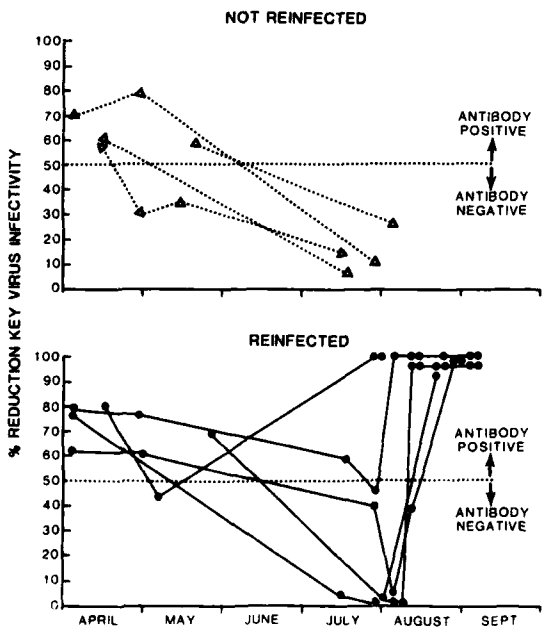

Fig. 2. Temporal fluctuation in KEY virus antibody prevalence of nine recaptured gray squirrels, 1975.

Table 2. KEY virus isolation rates for *Ae. atlanticus* collected in PCS, 1975

Collection dates[a]	No. tested (no. pools)	Avg no./ trap-night	No. viral isolates (no. identified)
22–26 July	18,450 (739)	461	50 (20)
27 July–5 August	24,450 (980)	611	68 (23)
6–15 August	11,175 (450)	279	33 (15)
16–25 August	3,100 (124)	78	11 (11)
26 August–4 September	1,350 (56)	34	5 (5)
5–14 September	1,025 (41)	26	1 (1)
15–24 September	2,575 (107)	64	6 (6)
25 September–4 October	1,200 (50)	30	7 (7)
5–14 October	800 (34)	20	9 (9)
15–23 October	375 (15)	9	1 (1)
Total	64,500 (2,596)	161	191 (98)

[a] Inclusive dates, total mosquitoes captured for 40 trap-nights

Table 3. KEY viral infection and transmission rates for *Ae. atlanticus* exposed to different concentrations of virus

Year[a]	Route of exposure	Viral dose[b]	Incubation days	Infection rate[c]	Transmission rate[d]
1975	Per os	1.5	21, 28	0 (15)	Not done
		1.9	5, 28	22 (51)	Not done
		2.8	21	54 (13)	Not done
		4.0	5, 7, 14, 21	95 (20)	0 (19)
		4.5	5, 7, 14, 21	100 (15)	0 (12)
1985	Per os	3.0	14, 21	48 (33)	0 (16)
		3.8	14, 21	100 (16)	0 (16)
		4.3	14, 21	100 (15)	0 (15)
		4.7	14, 21	100 (4)	25 (4)
1985	Intrathoracic	2.0	16	100 (46)	72 (46)

[a] Mosquitoes not available for other years because of infrequent emergence and/or breeding site destruction associated with lumbering and reforestation.
[b] Log_{10} $SMICLD_{50}$/ml (1975), PFU/ml (1985), mean titers determined for five individual mosquitoes immediately after they ingested or were inoculated with virus.
[c] Percentage of mosquitoes infected (no. exposed to virus).
[d] Percentage of mosquitoes that transmitted virus (no. fed upon mice).

amount of virus ingested increased from 1.9 to 4.5 log_{10} $SMICLD_{50}$/ml, the infection rate increased from 22 to 100%. However, a lethal dose of KEY virus was not transmitted to mice by 31 infected mosquitoes during the 1975 experiments, and only 1 of 51 mosquitoes infected per os transmitted a lethal viral dose to mice during the 1985 experiment. KEY virus antibody was not detected in the blood of 43 of the remaining 50 mice that survived.

Evidence of KEY virus infection was seen in individual *Ae. atlanticus* that ingested 4.0 log_{10} $SMICLD_{50}$ of virus. The mean titer for three to five individual mosquitoes ranged from 3.3 and 3.8 log_{10} $SMICLD_{50}$ on incubation days 0 and 3, respectively, and then increased to $\geq$4.3 log_{10} $SMICLD_{50}$ on days 5, 7, 14, and 21.

Parenteral Infection and Transmission. Keystone virus was recovered from each of 46 *Ae. atlanticus* after inoculation, and 72% (n = 46) transmitted a lethal dose of virus to mice after 16 d incubation (Table 3). Evidence of KEY virus antibody was not detected in sera of mice fed upon by 7 of the remaining 13 infected mosquitoes.

Viral Titers and Transmission. The amount of KEY virus recovered from a sample of the parenterally infected *Ae. atlanticus* that transmitted virus to mice was comparable to viral titers observed for a sample of parenterally and per os-infected mosquitoes that did not transmit virus (Table 4). Comparable viral titers also were found in the thorax–abdomen and salivary gland; however, the glands of all parenterally infected mosquitoes yielded virus, whereas virus was detected in glands of only two of six per os-infected mosquitoes (Table 5).

Transovarial Infection and Transmission. A summary of transovarial transmission (TOT) and filial infection rates for KEY virus-infected *Ae. atlanticus* is presented in Table 6. Although first- and second-ovarian cycle eggs were obtained from 38 infected females, eggs from only 13 females hatched. Seven of the 13 (54%) females transmitted KEY virus to progeny. The filial infection rate for the first ovarian cycle was 20% (n = 35), but increased substantially to 76% (n = 33) in the second-ovarian cycle. The overall filial infection rate for the two ovarian cycles was 47% (n = 68). One of two transovarially infected females transmitted a lethal dose of KEY virus to a suckling mouse.

Table 4. KEY viral titers and virus transmission capability for *Ae. atlanticus* after different routes of exposure

Route of infection	No. infected mosquitoes	Viral titers (log_{10} PFU/ml)	Transmission
Intrathoracic	10	4.5[a]	10/10[b]
Intrathoracic	6	4.3	0/6
Per os	10	4.1	0/10

[a] Mean titer.
[b] Number of mosquitoes that transmitted virus/number fed upon mice.

Discussion

The emergence and abundance of *Ae. atlanticus* varies from year to year depending on the amount of rainfall (LeDuc et al. 1975c). Sufficient rain fell during July 1975 to allow for the emergence of an exceptionally large population of this species. Subsequently, newly emerged and presumably trans-

Table 5. KEY viral titers in the thorax-abdomen and salivary glands of *Ae. atlanticus* in relation to this species' transmission capability after different routes of infection

Route of infection	No. infected mosquitoes	Viral titers[a]		Virus transmission
		Thorax/abdomens	Salivary glands	
Intrathoracic	6	4.0	2.5	6/6[b]
Intrathoracic	6	4.5	2.6	0/6
Per os	6	4.2	3.3[c]	0/6

[a] Mean viral titer (log_{10} PFU/ml).
[b] No. mosquitoes that transmitted virus/number fed upon mice.
[c] Keystone virus detected in salivary glands in two of six mosquitoes.

Table 6. Summary of transovarial transmission (TOT) rates for *Ae. atlanticus* infected parenterally with KEY virus

	1st ovarian cycle				2nd ovarian cycle				Total
	Larvae	♂♂	♀♀	All	Larvae	♂♂	♀♀	All	
TOT positive (n = 7)	6/34[a] (18)	—	1/1 (100)	7/35 (20)	15/22 (68)	4/4 (100)	6/7 (86)	25/33 (76)	32/68 (47)
TOT negative (n = 6)	0/31	0/9	0/11	0/51	0/3	0/1	0/1	0/5	0/56

[a] No. virus-positive mosquitoes/number tested (% positive).

ovarially infected *Ae. atlanticus* transmitted KEY virus to gray squirrels. Virus transmission was extensive with 65% (n = 43) of the seronegative squirrels having a KEY viral infection between the latter halves of July and September 1975. Other mosquito species that also attained peak population densities after heavy rains in the PCS included *Aedes canadensis* (Theobold), *Culex salinarius* Coquillett, and *Psorophora ferox* (von Humboldt) (Saugstad et al. 1972), but these species have not been associated with KEY virus except for a single isolation from *Ae. canadensis* (LeDuc et al. 1975c).

Evidence of KEY virus infection was found in only a single squirrel during 1976 and none during 1977 and 1978. The decline in virus transmission was associated with moderate rainfall that resulted in the emergence of low numbers of *Ae. atlanticus* in 1976 and 1978 and no apparent emergence during 1977. These data illustrate the importance of sampling vertebrate and vector populations over an extended time to determine the prevalence as well as the ecology of arboviruses. For example, data for 1978 indicated that the gray squirrel rarely was infected with KEY virus and, except for 1975, *Ae. atlanticus* was uncommon in the study area.

Serological evidence of KEY viral infection in the gray squirrel population was observed from 1975 through 1978, but virus transmission to squirrels was limited to 1975 and 1976 and only during the presence of adult *Ae. atlanticus*. Antibody observed in squirrels before the emergence of this mosquito in 1975 apparently was acquired during or before 1974. Seroconversions were not demonstrated, and most of the captured squirrels were born before 1975. Virus activity in the PCS before 1975 was evident by the collection of KEY virus-infected *Ae. atlanticus* from 1971 through 1974, and the virus was transmitted to sentinel rabbits (LeDuc et al. 1975b, LeDuc 1978). These data support previous observations that implicated *Ae. atlanticus* as the primary vector of KEY virus in the PCS (LeDuc et al. 1975c), and documented that a high proportion of gray squirrels had a KEY viral infection before the transmission of KEY virus to squirrels during 1975.

Experimental infection of gray squirrels with KEY virus produced a viremia with viral titers that exceeded the theoretical threshold required to infect *Ae. atlanticus* (Watts et al. 1979). These data implied that infected squirrels were capable of amplifying the prevalence of KEY virus in the *Ae. atlanticus* population. However, evidence of a viremia was not detected in squirrels sampled during 1975, including six squirrels that seroconverted only 4–6 d after blood was obtained for virus assay. Data suggested that the majority of the captured squirrels, mainly adults, had a previous KEY viral infection, and therefore antibody may have prevented the development of a viremia. Although the viremic response of these squirrels to a second KEY viral infection is unknown, experimental data indicate that preexisting KEY viral antibody suppresses the development of a second viremia in the Eastern cottontail rabbit (*Sylvilagus floridanus*) (Watts et al. 1979). Our failure to isolate virus from squirrels does not appear to be related to the assay system, because KEY virus was detected readily in the blood of experimentally infected gray squirrels and in *Ae. atlanticus* by the same technique.

Although our data failed to portray a readily discernible KEY virus amplifying role for infected gray squirrels, the MIR for this virus in the *Ae. atlanticus* populations increased following both the first and second emergence of this mosquito during 1975. A similar pattern of increase in the MIRs for KEY virus in *Ae. atlanticus* in the PCS (LeDuc et al. 1975c) and for California encephalitis virus in *Ae. melanimon* (Dyar) in Sacramento Valley, California (Turell et al. 1982) has been reported. Although our observations are likely to reflect virus recruitment into the mosquito population, the mechanism or mechanisms, as illustrated by this study, will not be readily determined under field conditions. For example, our data tend to argue against virus amplification by gray squirrels, but they also suggest that our trapping technique was biased toward the capture of the same individuals of the older cohort, or squirrels more likely to have experienced a previous KEY viral infection. As described by Flyger (1955), adult squirrels monopolize food supplies, such as baited traps and feeding stations used to capture squirrels in this study. The high recapture rate of 89% (n = 71) for individual adult squirrels during this study (1975) at the same or nearby trapping sites corroborates this observation (D.M.W., unpublished data). Thus, it is possible that the immature cohorts, or the squirrels more likely to serve as amplifying hosts were seldom captured because of this behavioral characteristic and, therefore, may have unknowingly contributed to the increased KEY virus MIR in the *Ae. atlanticus* population.

Other mammalian species, such as cottontail rabbits, cannot be excluded as viral amplifying hosts. Experimental data revealed that this species developed a viremia following inoculation with KEY virus, and limited serosurveys demonstrated evidence of natural KEY virus infections (Watts et al. 1979). In addition, other variables such as the possibility of venereal transmission cannot be excluded as influencing the observed ratio of KEY viral-infected to uninfected mosquitoes.

The present data reinforce earlier observations that transovarial transmission is an overwintering mechanism for KEY virus in the PCS (LeDuc 1978). Isolation of this virus from the first newly emerged *Ae. atlanticus* and serological evidence of KEY viral infections in gray squirrels only 10 d after the emergence of this mosquito species suggest that the transovarially infected females are capable of transmitting KEY virus. This was the first evidence that presumably transovarially infected *Ae. atlanticus* transmitted this virus to a free-ranging wild mammal, and it is consistent with previous data that recently emerged *Ae. atlanticus* transmitted KEY virus to caged domestic rabbits (LeDuc 1978).

Although serological reactivity to JC virus was detected in gray squirrels, PRN_{50} titers suggested that squirrels were infected with only KEY virus. Apparently, neutralization of JC virus infectivity was due to the crossreactivity of KEY virus antibody. These viruses are related antigenically (Sather & Hammon 1967) and a similar pattern of cross-reactivity was demonstrated previously between JC and KEY viruses (Watts et al. 1979, Watts et al. 1982).

The KEY virus infectious $dose_{50}$ (ID_{50}) for *Ae. atlanticus* was approximately 3.0 $\log_{10}$ $SMLD_{50}$, or PFU, in comparison with 4.9 $\log_{10}$ $SMLD_{50}$ for *Ae. canadensis* (D.M.W., unpublished data), and 5.2 $\log_{10}$ $SMLD_{50}$ for *Psorophora ferox* (Roberts 1973). On the basis of viremia levels found in KEY virus-infected gray squirrels (Watts et al. 1979), *Ae. atlanticus* would become readily infected; however, the lower susceptibility of *Ae. canadensis* and *Ps. ferox* reduce the vector potential of these species. In addition, the host preference of *Ae. canadensis* may preclude frequent contact with the principal vertebrate host for this virus. LeDuc et al. (1972) found that this species feeds primarily on white-tailed deer (*Odocoileus virginianus*) and reptiles in the PCS, whereas *Ae. atlanticus* feeds preferentially on white-tailed deer, domestic goats, and gray squirrels.

Our observations indicate that per os-infected *Ae. atlanticus* seldom transmit KEY virus to mice, whereas mosquitoes infected by the parenteral route are efficient transmitters. Limited data suggest that the failure of per os-infected mosquitoes to transmit virus is due to a midgut escape or other infection barriers associated with the salivary glands. The latter was suggested by the failure to detect virus in glands of four of six infected mosquitoes and the absence of detectable antibody in mice fed upon by infected mosquitoes. While it is possible that 1- to 3-d-old mice are not immunologically competent, data (D.M.W., unpublished data) indicate that mice have a lethal infection after subcutaneous inoculation with 100 PFU or greater doses of KEY virus. Attempts to detect antibody in mice that survived sublethal doses of KEY virus were unsuccessful. Thus, the failure of KEY virus-infected *Ae. atlanticus* to transmit virus to mice appears to resemble closely the laboratory data reported for La Crosse virus-infected *Ae. hendersoni* (Cockerell). In spite of high infection rates, horizontal virus transmission rates for this species were low, ranging from 0 to 14% (Watts et al. 1975, Grimstad et al. 1985), and La Crosse virus was transmitted vertically by this mosquito (S. L. Paulson, personal communication).

The observation that a transovarially infected female transmitted KEY virus to a mouse is consistent with a previous report (LeDuc 1978) that newly emerged transovarially infected *Ae. atlanticus* are capable of transmitting KEY virus to vertebrates in the field. Furthermore, vertically infected, rather than per os-infected, *Ae. atlanticus* may account for the high prevalence of KEY viral infection in the gray squirrel population observed on the DelMarVa Peninsula. Therefore, even if a gray squirrel circulates a mosquito infectious dose of KEY virus, further transmission to other vertebrates may be precluded by the failure of per os-infected mosquitoes to transmit an infectious viral dose. Additional field and laboratory studies are needed to determine whether this observation is operative under field conditions.

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